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Binding Sites for Ca^{2+}-Channel Effectors and Ryanodine in *Periplaneta americana*—Possible Targets for New Insecticides†

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Abstract: The calcium channel and the 'calcium release channel' of muscle membrane of the cockroach *Periplaneta americana* have been characterized. Biological assays with calcium channel blockers and ryanodine on different insects and acari revealed pronounced insecticidal effects with ryanodine, but not with calcium channel blockers, at concentrations between 0·1 and 300 $\mu g\ ml^{-1}$. Skeletal muscle membranes derived either from the tubular network or from the sarcoplasmatic reticulum of *P. americana* were characterized with respect to the binding of the dihydropyridine (DHP) [^{3}H]isradipine (PN 200-110), the phenylalkylamine [^{3}H]verapamil and the alkaloid [^{3}H]ryanodine. Preliminary binding studies with the benzothiazepine [^{3}H]diltiazem suggest a low-affinity binding site with a IC_{50} value of 3·3 μM. All binding sites tested were sensitive to treatment with proteinase K. Optimal conditions for binding of the radioligand ryanodine revealed the highest specific binding at pH 8 and at calcium chloride concentrations between 100 and 500 μM. EGTA at 10 μM abolished 95% of the ryanodine binding. Binding studies with calcium channel binding sites revealed a pronounced effect of low Ca^{2+} concentrations on specific isradipine binding, whereas verapamil and diltiazem binding were only reduced by the presence of 200 μM EGTA. With respect to high Ca^{2+} concentrations, specific binding of diltiazem, isradipine and verapamil was reduced by 73, 40 and 20%, respectively, at 5 mM Ca^{2+}.

Radioligand binding experiments showed high-affinity binding sites for ryanodine and isradipine. K_D values of 0·95 nM (B_{max} = 550 fmol mg^{-1} protein) and 0·75 nM (B_{max} = 213 fmol mg^{-1} protein) were determined respectively. A lower-affinity binding site was identified in binding studies with verapamil (K_D = 7·4 nM and B_{max} = 27 fmol mg^{-1} protein). [^{3}H]isradipine displacement studies with several dihydropyridines revealed the following ranking of affinity: nitrendipine > isradipine > Bay K8664 ≫ nicardipine. Displacement of [^{3}H]verapamil binding by effectors of the phenylalkylamine binding site showed that bepridil and *S*(−)verapamil had the highest affinities of the compounds tested followed by (±)verapamil, nor-methylverapamil and *R*(+)verapamil.

Key words: L-type Ca^{2+}-channel, ryanodine receptor, *Periplaneta americana*, ryanodine, verapamil, isradipine (PN 200-110), diltiazem, *Boophilus microplus*

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1 INTRODUCTION

In vertebrate muscle membrane preparations a variety of different Ca^{2+} channel types (N-, T- and L-type) have been classified on the basis of their electrophysiological and pharmacological properties.[1] The α_1 sub-unit of the L-type dihydropyridine (DHP) receptor is a target for three classes of Ca^{2+} entry blockers such as dihydropyridines, phenylalkylamines and benzothiazepines. Examples from the three groups of Ca^{2+} channel blockers are isradipine (PN 200-110), verapamil and diltiazem. Inhibition of Ca^{2+} entry through voltage-dependent Ca^{2+} channels by specific organic calcium channel inhibitors represents one of the most important therapeutic means of treatment of many cardiovascular disorders such as angina, hypertension and tachycardia.[2] Much less is known of Ca^{2+} channel diversity and ryanodine receptors in invertebrates. Whereas Ryania, a plant alkaloid, containing the active principles ryanodine and 9,21-didehydroryanodine, has been well-known as a botanical insecticide for more than fifty years,[3] calcium channels in arthropods represent an additional, yet unexploited, potential target site for new insecticides.

Pauron *et al.*[4] have studied the interaction of putative Ca^{2+} channels of *Drosophila melanogaster* Meig head membranes with molecules of the phenylalkylamine series. Pelzer *et al.*[5] identified and characterized the binding sites for phenylalkylamines and 1,4-dihydropyridines in *Drosophila* brain membranes using binding studies and affinity and immunoblot techniques.

Another study characterized the phenylalkylamine binding site in nervous system and muscle membranes of *Periplaneta americana* L.[6] The biological effects of certain calcium channel blockers such as diltiazem and isradipine on invertebrates have not yet been examined.

The calcium channel complex in vertebrates is located mainly in the transverse tubules, where it is closely associated with the ryanodine-sensitive Ca^{2+} release channel (ryanodine receptor) in the terminal cisternae of the sarcoplasmatic reticulum. Upon membrane depolarization, a rapid conformational change in the α_1-subunit induces the opening of the 'calcium release' channel *via* a fast, still unknown, mechanism, thereby triggering muscle contraction.[7] The 'calcium release' channel was identified as a ryanodine receptor that can bind the ligand ryanodine. Ryanodine was first reported in 1945 by Pepper and Carruth[7] and was characterized after isolation and crystallization from the ground stem wood and root of *Ryania speciosa* Vahl by Rogers *et al.* in 1948.[8] The ryanodine receptor appears to have homotetrameric structure, and forms, with the four subunits, a hydrophilic pore in the sarcoplasmatic reticulum. The large molecular mass of the ryanodine receptor (~450 kDa) and its association as a homotetramer gives the receptor its characteristic structure and allows rapid migration through a sucrose gradient.[9]

The biological effect of ryanodine on insects (*Musca domestica* L. and *P. americana*) was described by Waterhouse *et al.*[10] who showed that ryanodine is active in insect systems following intrathoracic injection or by oral administration. Furthermore, a specific ryanodine binding site was described recently in membrane preparations of insects.[11]

This paper reports a calcium channel system, as well as a ryanodine receptor, in *P. americana* and introduces a binding assay suitable for screening of ryanodine receptor and Ca^{2+} channel effectors.

2 EXPERIMENTAL METHODS

2.1 Materials

[^{3}H]Ryanodine (62·8 Ci mmol^{-1}), [^{3}H]verapamil (62·3 Ci mmol^{-1}), [^{3}H]diltiazem (85·5 Ci mmol^{-1}) and [^{3}H]isradipine (PN 200-110; methyl isopropyl (±)-4-(4-benzofurazanyl)-1,4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylate; 85·8 Ci mmol^{-1}) were purchased from Dupont NEN Research and unlabelled ryanodine, (±)verapamil HCl, *S*(−)verapamil HCl, *R*(+)verapamil HCl, bepridil HCl, (±)nor-methylverapamil, loperamide HCl, nitrendipine, R(+)-Bay K8644, nicardipine HCl and diltiazem HCl were obtained from RBI (MA, USA). Isradipine was kindly supplied by Sandoz Pharma AG, Basel, Switzerland (Dr G. Engel and Dr H. Widmer).

2.2 Methods

2.2.1 Insects

Adult male and female cockroaches (*P. americana.*, normal susceptible strain) were dissected to obtain the femoral and thoracic muscle after digestive tracts were removed. These were placed in buffer A containing the protease inhibitors aprotinin, leupeptin and pepstatin (4, 4 and 3 μg mg^{-1}, respectively) and were immediately frozen in liquid nitrogen. Prior to use, the insect preparations were stored for a minimum of one day at −80°C.

2.2.2 Membrane preparations

Two buffers were used: (A) 50 mM Tris HCl, pH 7·4 and (B) 20 mM Tris HCl, 1·5 M KCl, 0·3 M sucrose, 500 μM $CaCl_2$, pH 8. Tris HCl buffers were adjusted at the experimental temperature. The thawed tissues in buffer A were homogenized with a Bühler homogenizator (6 × 30-s burst at 24000 rev min^{-1}). With verapamil, diltiazem and isradipine, the homogenate was filtered through two layers of mull (muslin) and five layers of cheesecloth and centrifuged (125000*g*; 45 min; 4°C). In the case of ryanodine, the homogenate was filtered

through two layers of mull and one layer of cheesecloth, centrifuged (20 min; 1950*g*), washed and the supernatant was centrifuged again (30 min; 30000*g*; 4°C). The pellets were resuspended in buffer B (containing the protease inhibitors listed above) and the protein concentrations were determined according to the method of Bradford[12] using bovine serum albumin as standard. Each experiment involved a new tissue preparation.

2.2.3 Binding assays

Incubation mixtures consisted of buffer B plus membrane preparation (0·5 mg protein ml^{-1}) and 11% by volume [^{3}H]radioligand stock solution in incubation buffer containing 10 g $litre^{-1}$ ethanol. Specific binding was determined as the difference between total binding ([^{3}H]radioligand) and non-specific binding ([^{3}H]radioligand plus 1000-fold excess of unlabelled ligand). Free ligand was separated from the protein-bound ligand by filtration through pre-wetted Whatman GF/F glass-fibre filters, followed by washing with ice-cold buffer B without protease inhibitors (2 × 5 ml). This washing procedure removed 96% of the free ligand. Each determination was repeated four times. The activity retained on the filters was measured using standard liquid scintillation counting techniques with Optifluor (Packard) as scintillation fluid and a Phillips PW 4700 liquid scintillation counter with 50% counting efficiency for tritium.

2.2.4 Measurement of association/dissociation kinetics

The association kinetics of [^{3}H]ryanodine, [^{3}H]verapamil and [^{3}H]isradipine binding were measured by filtration at different times after addition of the membrane preparation. Dissociation of [^{3}H]radioligand from the receptor equilibrium complex was determined by incubation of [^{3}H]radioligand with membranes followed by addition of unlabelled ligand (1000-fold excess) to a portion of the incubation mixture; displacement of radioligand was investigated by determination of residual specific binding after further incubation for different times.

2.2.5 Binding studies with ryanodine and calcium channel effectors after heat treatment, incubation with DDT or proteolytic digest of membrane preparations

Membrane protein (0·5 mg ml^{-1}) prepared in protease-free buffer was pre-incubated at 29°C, pH 8, for 60 min with 0·5 mg ml^{-1} proteinase K, 5 mg ml^{-1} trypsin and 5 mg ml^{-1} dithiotreitol (DTT) respectively, prior to standard binding assay with [^{3}H]radioligand as described in Section 2.2.3 and indicated in the figures. Heat treatments were carried out for 45 min at 60°C. The ryanodine receptor was pre-incubated with 0·02 mg ml^{-1} proteinase K for 60 min. Specific binding was measured at equilibrium as described in the text.

2.2.6 Displacement of [^{3}H]isradipine, [^{3}H]verapamil and [^{3}H]diltiazem binding by calcium channel effectors

Muscle membrane protein (0·5 mg ml^{-1}) was incubated with radioligand as described in Section 2.2.3 and indicated in the figures. Radioligand binding was displaced by increasing concentrations of unlabelled compounds ranging from 1 pM to 100 μM. K_i values were calculated from four-parameter logistic curve-fitting data corrected according to the method of Cheng and Prussoff.[13]

All graphs were drawn and calculated using curve fitting programs from Sigma Plot Scientific Graphic Software (Jandel Scientific).

2.3 Insecticidal activities

Approximately 50 *Lucilia cuprina* Wied. larvae (first instar) were treated with calcium channel blockers or ryanodine added to minced horse meat in concentrations ranging from 0·1 to 300 μg ml^{-1}. LD_{50} values (dose giving 50% kill) were determined after 24 and 48 h (larval mortality) and at day 12 (counting for emerged adults). Mortality of adult *L. cuprina* was assayed on filter disks. The flies were immobilized by carbon dioxide and transferred onto filter discs impregnated with verapamil, diltiazem, isradipine or ryanodine in concentrations ranging from 0·1 to 300 μg ml^{-1}. Fifth-instar larvae of *P. americana* (average weight 840 mg per animal) were treated by feeding (oral administration of 1 μl solution in dimethylsulfoxide with a Hamilton 10-μl glass syringe) with a ryanodine, verapamil or diltiazem solution at concentrations ranging from 5 mg to 20 g $litre^{-1}$. LD_{50} values were determined after 24 and 72 h and at days 7 and 14 counting the living insects.

The effect of calcium channel blockers and ryanodine on acari was tested by treatment of adult *Amblyomma hebraeum* Koch and engorged female *Boophilus microplus* Can. by dipping or injection with the test compound, respectively. Concentrations ranged from 1 to 3000 μg ml^{-1} (dipping) and doses ranged from 0·02 to 20 μg $tick^{-1}$ (injection). The average weight of injected *B. microplus* was 310 mg per animal. With *Amblyomma* the efficacy was determined after 1 and 24 h by counting living adult ticks on a 40°C heating table. With *Boophilus*, egg-laying and larval hatching was determined after one and four weeks, respectively.

The efficacy of calcium channel blockers was tested on fleas (*Ctenocephalides felis* Bche.). Verapamil and diltiazem were added to cattle blood at 100 μg ml^{-1}. The fleas (in 27°C flea feeding chambers closed with gauze) were allowed to feed from the blood (heated to 37°C) through parafilm membranes for 24 h. Mortality of fleas was evaluated after 24 h ingestion.

Fifty percent lethality or efficacy values were obtained from probit analyses.[14]

3 RESULTS

3.1 Effect of calcium on [^{3}H]ryanodine binding

Calcium is an essential divalent cation for ryanodine binding. The rate of [^{3}H]ryanodine binding increased with increasing Ca^{2+} concentration, with a maximum at 100–500 μM calcium. However at 0·5 mM binding decreased, becoming constant between 4 and 25 mM (Fig. 1(a)).

3.2 Effect of pre-incubating calcium with EGTA or EDTA on [^{3}H]ryanodine binding

Incubation of muscle membranes with EGTA, but without Ca^{2+}, in buffer indicated a decrease in specific binding of [^{3}H]ryanodine. When 10 μM EGTA was added to the incubation buffer, the specific [^{3}H]ryanodine binding declined by 95%. The chelation

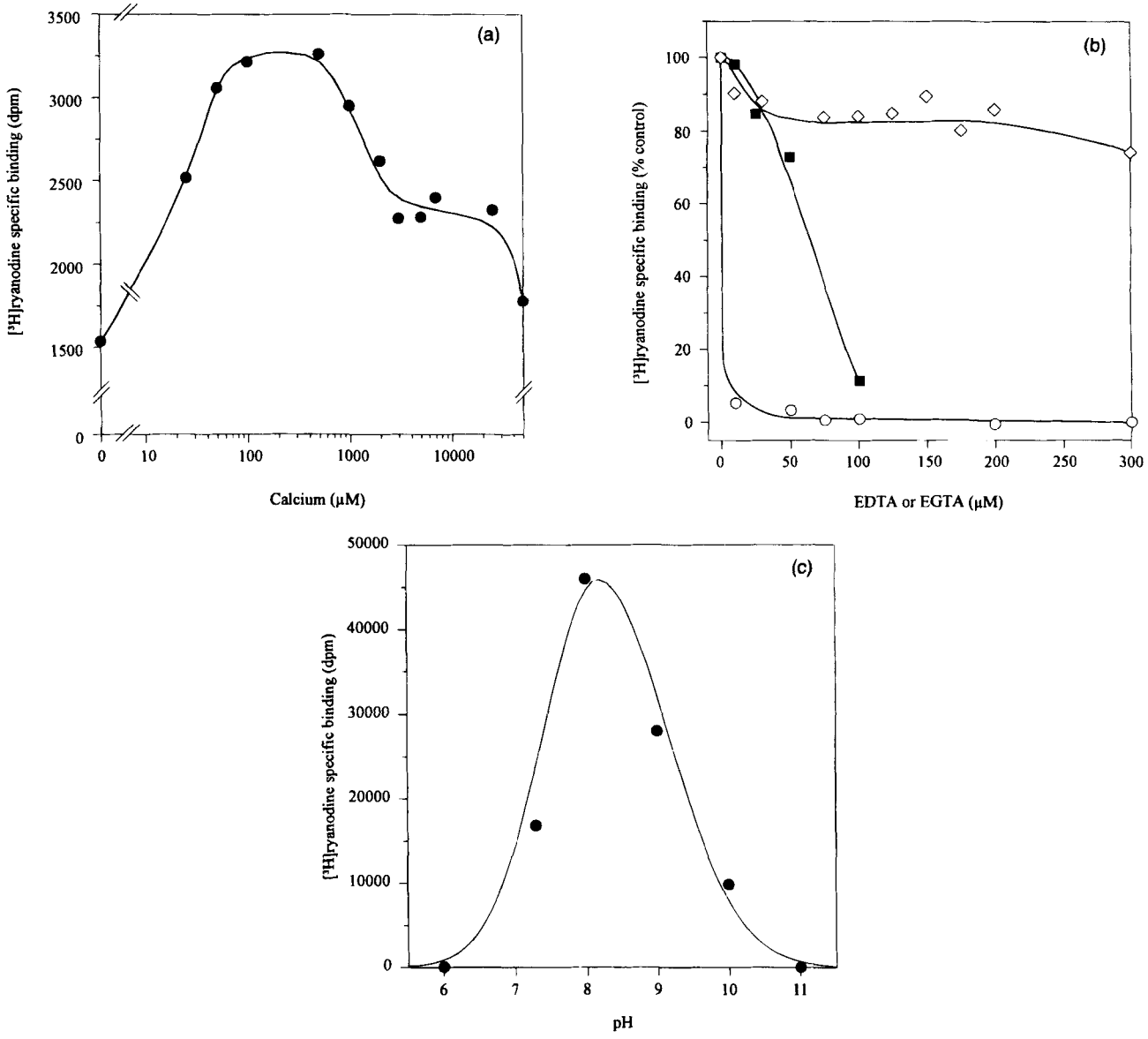

Fig. 1. (a) Effect of calcium on [^{3}H]ryanodine binding to skeletal muscle membranes of *Periplaneta americana*. 5 nM [^{3}H]ryanodine was incubated 70 min at 33°C with 0·5 mg ml^{-1} protein in buffer B and different concentrations of calcium. (b) Effect of EDTA and EGTA on [^{3}H]ryanodine binding to skeletal muscle membranes of *Periplaneta americana*. 3 nM [^{3}H]ryanodine was incubated (90 min; 27°C) with 0·5 mg ml^{-1} protein in buffer B. (◇) Effect of EGTA after preincubation with 100 μM calcium (100% corresponds to 4593 dpm). (■) Effect of EDTA on [^{3}H]ryanodine binding (0·35 mg ml^{-1} protein; 100% corresponds to 2484 dpm). (○) Effect of EGTA (without calcium in the incubation buffer) on [^{3}H]ryanodine binding (100% correspond to 3746 dpm). (c) pH dependence of [^{3}H]ryanodine binding to skeletal muscle membranes of *Periplaneta americana*. 5 nM [^{3}H]ryanodine was incubated (70 min; 27°C) with 0·5 mg ml^{-1} protein. Buffers were phosphate buffer (pH 6–7·4), Tris (pH 7·4–8), boric acid buffer (pH 8–10), sodium hydroxide/glycine buffer (pH 10–11).

of divalent cations other than calcium with EDTA resulted in a 90% decrease in binding but only at concentrations of >100 μM. A short pre-incubation of the membrane protein with 100 μM calcium and 3 nM [^{3}H]ryanodine showed no decrease in specific [^{3}H]ryanodine binding after addition of EGTA (Fig. 1(b)).

3.3 Effect of monovalent cations on [^{3}H]ryanodine binding

The ryanodine receptor, which interacts with the L-type calcium channel in the T-tubule membrane of muscles, depends on different monovalent and divalent cations. [^{3}H]ryanodine binding increased with an increase in K^+ or Na^+ concentration in the incubation buffer, the rate of increase being the same for K^+ and Na^+ at concentrations up to 1 M. At Na^+ concentrations $>1{\cdot}25$ M, [^{3}H]ryanodine binding showed no further increase, in contrast to incubation buffer with K^+, where a steady increase at concentrations up to 2 M was observed (data not shown).

3.4 Effect of pH on [^{3}H]ryanodine binding

Greatest [^{3}H]ryanodine binding occurred at pH 8 (Fig. 1(c)) and binding did not occur at pH <6 and or pH >11. At physiological pH (7·4) binding was only 26% of that achieved at pH 8, at which binding was at a maximum.

3.5 Effect of pre-treatment of membrane preparations from *P. americana* with heat proteases and DTT on ryanodine, isradipine, verapamil and diltiazem binding

Table 1 summarizes our results. After treatment with 0·5 or 0·02 mg ml^{-1} proteinase K for 1 h, no significant specific binding was detectable with 1,4-dihydropyridine (1,4-DHP) and ryanodine binding sites. With verapamil and diltiazem binding sites, only 22% and 57%, respectively, of the binding in the control was detectable after pre-treatment with 0·5 mg ml^{-1} proteinase K. Pre-treatment with 5 mg ml^{-1} trypsin abolished 87% and 59·4% of specific verapamil and diltiazem binding, whereas no effect of trypsin was measured with the 1,4-DHP binding site. All calcium channel binding sites tested were sensitive to dithiothreitol. A reduction of 75, 57 and 32% of the specific binding was observed with verapamil, diltiazem and isradipine binding sites respectively. While heat treatment of membrane preparations at 60°C reduced specific binding with 1,4-DHP and benzothiazepine binding sites by nearly 90% and >60%, respectively, specific phenylalkylamine binding was not affected under these conditions.

3.6 Effect of calcium on ligand binding by L-type calcium channel binding sites

Different Ca^{2+} concentrations and Ca^{2+} trapping by EGTA were tested with isradipine, verapamil and diltiazem binding sites from muscle membrane preparations of *P. americana* (Table 1). In presence of EGTA (200 μM)

TABLE 1
Degradation of the Calcium Channel and Ryanodine Binding Sites by Chemical, Enzyme, Proteinase K or Heat Treatment

	Specific binding (% control ± S.D.)			
Conditions	[^{3}*H*]*isradipine*	[^{3}*H*]*verapamil*	[^{3}*H*]*diltiazem*	[^{3}*H*]*ryanodine*
Control	100 (±8·8)	100 (±4·1)	100 (±5·5)	100 (±3·7)
Proteinase K	0 (±4·1)	22·1 (±3·0)	57·5 (±6·5)	1·6 (±2·3)
Trypsin	111 (±9·6)	12·9 (6·8)	40·4 (±8·4)	n.d.
DTT	68·5 (±5·4)	25·3 (±7·0)	43 (±5·8)	n.d.
60°C	10·7 (±5·2)	102·9 (±4·3)	38·3 (±6·7)	n.d.
0 μM Ca^{2+}	44·6 (±8·7)	101·3 (±3·4)	141·6 (±3·5)	in Fig. 1(a)
5000 μM Ca^{2+}	59·6 (±3·7)	80·4 (±2·9)	31·1 (±2·7)	in Fig. 1(a)
200 μM EGTA	51·3 (±4·4)	57·6 (±5·1)	12·3 (±5·5)	in Fig. 1(b)

Periplaneta americana membrane protein (0·5 mg ml^{-1}) was incubated at 29°C, pH 8, for 60 min with 0·5 mg mg^{-1} proteinase K and 5 mg mg^{-1} trypsin or DTT respectively, then incubated for indicated times with [^{3}H]radioligand. Heat treatment was carried out for 45 min at 60°C. Effect of calcium on calcium channel binding sites of muscle membranes of *P. americana* was demonstrated. 1 nM [^{3}H]isradipine, 10 nM [^{3}H]verapamil or 250 nM [^{3}H]diltiazem was incubated 210, 40 and 30 min respectively at 29°C with 0·5 mg ml^{-1} protein in buffer B and different concentrations of calcium or 200 μM EGTA, respectively. The specific binding was measured at equilibrium as described in the text. Results are expressed as percentage of control. Control specific binding corresponds to 2213, 6463, 3065, 2465 dpm [^{3}H]ryanodine, [^{3}H]isradipine, [^{3}H]verapamil and [^{3}H]diltiazem binding, respectively. S.D.: standard deviation of mean values. n.d.: not determined.

the specific binding of [^{3}H]diltiazem, [^{3}H]isradipine and [^{3}H]verapamil was reduced by 88, 49 and 43% compared with that in the control, respectively. Specific binding in incubation buffer without supplemented Ca^{2+} was significantly reduced with isradipine binding sites. High Ca^{2+} concentrations (5 mM) reduced specific binding of [^{3}H]diltiazem, [^{3}H]isradipine and [^{3}H]verapamil by 69, 41 and 20% compared with the control (Table 1).

3.7 Saturation binding experiments of ryanodine receptor and L-type calcium channel binding sites

Scatchard analysis[15] of the saturation isotherm of [^{3}H]ryanodine binding to muscle membrane of *P. americana* indicated a high-affinity binding site (K_D = 0·95 nM and B_{max} of 550 fmol mg^{-1} protein) and single populations of binding sites (Fig. 2(a)). A Hill coefficient not significantly different from 1 (slope = 1·01) was obtained which suggested a single population of binding sites with the same affinity for this ligand (data not shown).

Scatchard analysis of the saturation isotherm of [^{3}H]isradipine binding also indicated a high-affinity binding site present at B_{max} of 213 fmol mg^{-1} protein and K_D = 0·75 nM (Fig. 2(b)). An equilibrium dissociation constant of 7·4 nM (Fig. 2(c)) and a B_{max} of 27 fmol mg^{-1} protein were determined for [^{3}H]verapamil binding to muscle membrane of *P. americana* with one population of binding sites.

3.8 Determination of rate constants for ryanodine receptor and L-type calcium channel binding sites

The association and dissociation rate constants of binding of [^{3}H]ryanodine, [^{3}H]isradipine and [^{3}H]verapamil to muscle membrane of *P. americana* were determined from the time course measurement. With association rate constants, the specific binding (B) was terminated at the indicated time intervals. The binding at equilibrium (B_e) was reached after 90, 210 and 40 min respectively (Fig. 3(a)–(c)). Transformation of the exponential curve from the time-dependent formation of the ligand-receptor-complex into the linear function ln ($B_e/(B_e - B)$) plotted versus time revealed the k_{obs} from the slope (inset Fig. 3(a)–(c)). The association rate constant results from $k^{+1} = [(k_{obs} - k^{-1})$/concentration of radioligand].

The dissociation rate constants (k^{-1}) for ryanodine, isradipine and verapamil calculated for the different receptor binding sites from the results presented in Fig. 4(a)–(c) were $3{\cdot}8 \times 10^{-3}$, $5{\cdot}1 \times 10^{-3}$ and $2{\cdot}7 \times 10^{-2}$ min^{-1}, respectively. The first order exponential decay curve of the dissociating complex was transformed into a linear function by plotting ln(B/B_0) (where B is the concentration of bound radioligand at an indicated time and B_0 is the concentration of bound radioligand at time zero) against time. k^{-1} is then given by the negative slope of the regression line (inset Fig. 4(a)–(c)).

3.9 Interaction of compounds with the 1,4-dihydropyridine, phenylalkylamine and benzothiazepine binding sites of the calcium channel and with the calcium release channel

A 40 + 60 mixture of 9,21-didehydroryanodine and ryanodine (corresponding to the usual *Ryania* extract) inhibited [^{3}H]ryanodine binding with a K_i value of $5{\cdot}02 \times 10^{-8}$ M. With pure ryanodine and 9,21-didehydroryanodine, K_i values of $7{\cdot}53 \times 10^{-8}$ M and $1{\cdot}95 \times 10^{-8}$ M, respectively, were determined from the displacement profiles (Fig. 5(a)).

All of the 1,4-dihydropyridines tested competed with the radiolabelled calcium antagonist [^{3}H]isradipine in

TABLE 2

Results of Binding Assays of the 'Calcium Release Channel' and the Calcium Channel in Muscle Membrane Preparations of the Cockroach *Periplaneta americana*

Ligand	*Association rate constant* (M^{-1} *min*$^{-1}$) (±*S.D.*)	*Dissociation rate constant* (*min*$^{-1}$) (±*S.D.*)	*Equilibrium dissociation constant* (*eq.*) (nM) (±*S.D.*)	*Binding sites* (B_{max}) (*pmol mg*$^{-1}$ *protein*) (±*S.D.*)	*Dissociation constant* (*kin.*) (nM)
[^{3}H]ryanodine (in Figs 2(a), 3(a), 4(a)	$7{\cdot}045 \times 10^6$ (±2.1)	$3{\cdot}77 \times 10^{-3}$ (±1·1)	0·95 (±0·1)	0·550 (±0·05)	0·54
[^{3}H]isradipine (in Figs 2(b), 3(b), 4(b)	$3{\cdot}24 \times 10^6$ (±1·8)	$5{\cdot}9 \times 10^{-3}$ (±1·1)	0·75 (±0·29)	0·213 (±0·03)	1·8
[^{3}H]verapamil (in Figs 2(c), 3(c), 4(c)	$7{\cdot}53 \times 10^6$ (±1·6)	$2{\cdot}72 \times 10^{-2}$ (±0·8)	7·4 (±1·03)	0·03 (±2·47)	3·53

The values for the equilibrium dissociation constants were calculated from saturation experiments and the kinetic dissociation constants were calculated from the rate constants: $K_D(kin) = k_{diss}/k_{ass}$. S.D.: standard deviation of the slope parameters.

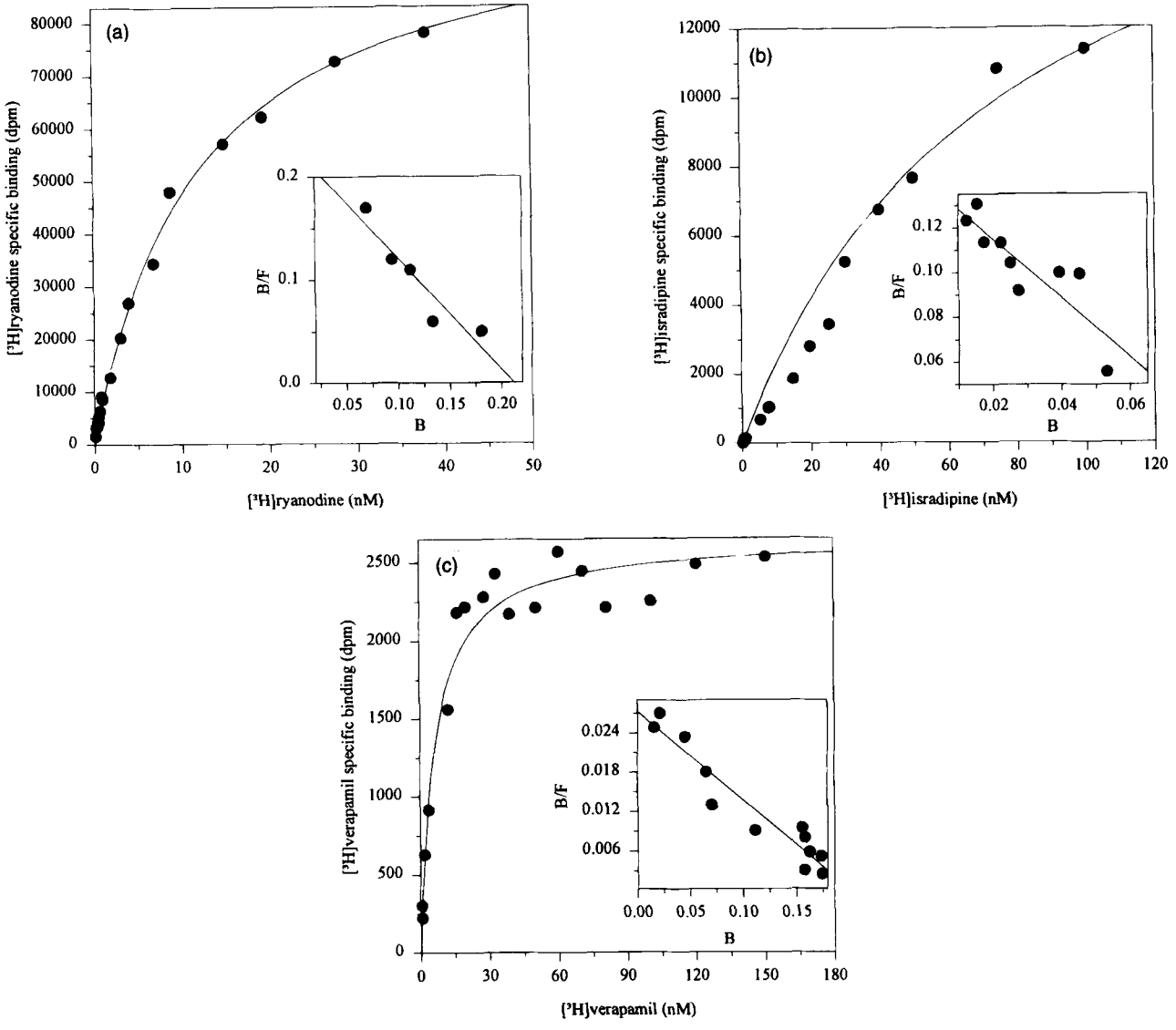

Fig. 2. Saturation kinetics shown as binding isotherms and Scatchard plot. (a) [^{3}H]ryanodine binding: incubation with 0·4 mg ml^{-1} thorax or muscle membrane protein for 90 min at 33°C using increasing amounts of [^{3}H]ryanodine. r^2 = 0·87. (b) [^{3}H]verapamil binding: incubation with 1 mg ml^{-1} thorax or muscle membrane protein for 40 min at 30°C using increasing amounts of [^{3}H]verapamil. r^2 = 0·915. (c) [^{3}H]isradipine binding: incubation with 0·5 mg ml^{-1} thorax or muscle membrane protein for 210 min at 30°C using increasing amounts of [^{3}H]isradipine r^2 = 0·74. Insets: B = specifically bound [^{3}H]radioligand; F = concentration of free radioligand. r^2: coefficient of correlation of least square fitted straight lines; standard deviation of the slope given in Table 2.

P. americana muscle membranes. K_i values of 2·3 × 10^{-9}, 1·2 × 10^{-9}, 7·7 × 10^{-9} and 1·1 × 10^{-7} M were determined for isradipine, nitrendipine, Bay K8644 and nicardipine, respectively (Fig. 5(b)).

Increasing concentrations of phenylalkylamine drugs inhibited [^{3}H]verapamil binding to muscle membranes (Fig. 5(c)). K_i values of 3·7 × 10^{-7} and 1·5 × 10^{-6} M were determined for (−)verapamil and (+)verapamil respectively. For the biologically active, *N*-demethylated metabolite of verapamil, (±)nor-methyl-verapamil and the non-selective calcium channel blocker, bepridil, K_i values of 9·7 × 10^{-7} and 4·3 × 10^{-7} M were determined from the displacement profiles.

Diltiazem inhibited [^{3}H]diltiazem binding to *P. americana* muscle membranes with an IC_{50} value of 3·3 × 10^{-6} M (Fig. 5(d)).

3.10 Biological assays

These were performed in order to evaluate whether the 'calcium release channel' and the calcium channel binding sites are susceptible to the action of ryanodine and calcium channel blockers in the arthropod target species. *L. cuprina* (first-instar larvae) were raised on minced horse meat containing varying amounts of ryanodine and calcium channel blockers, respectively. A

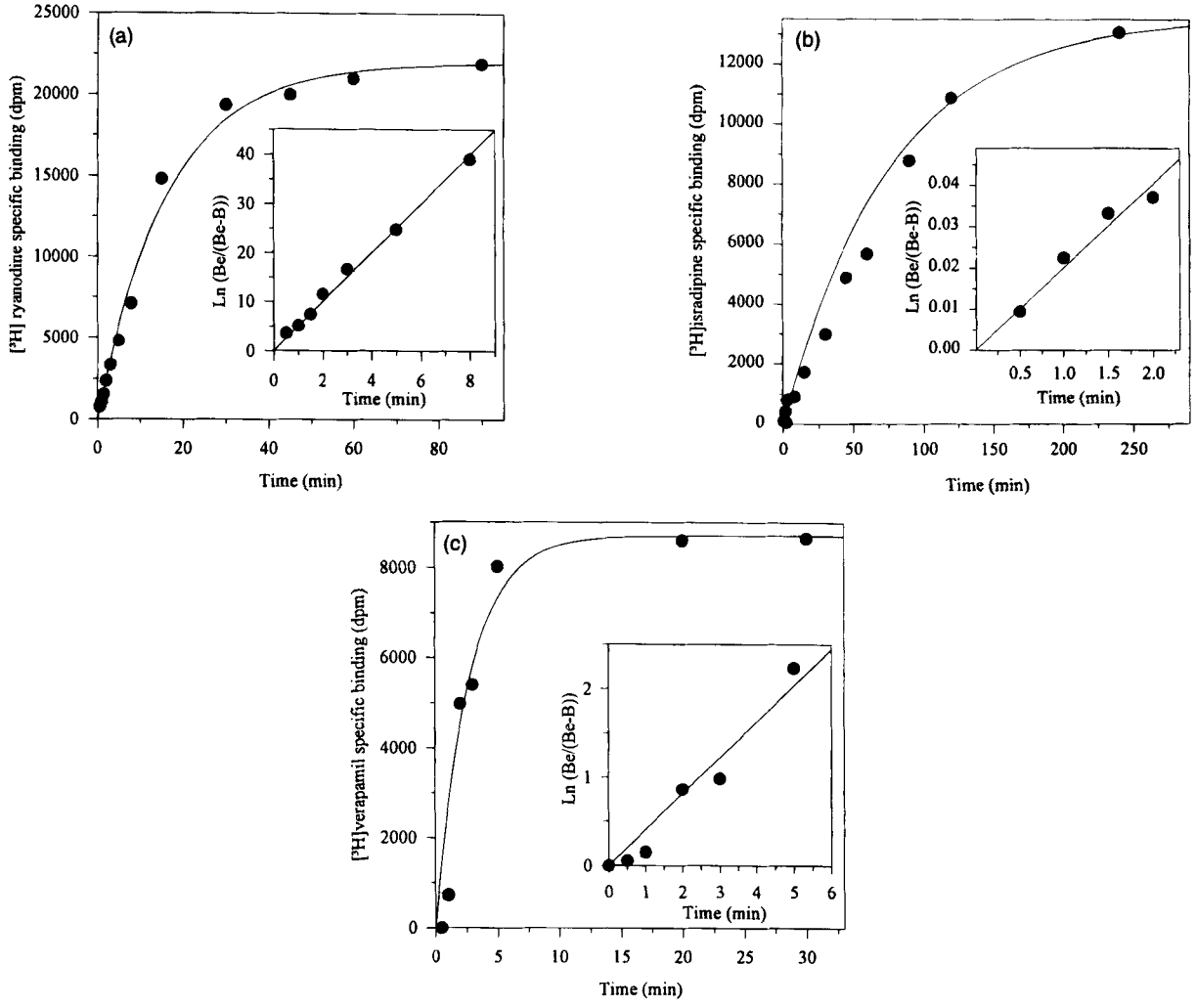

Fig. 3. Determination of association rate constants. (a) Association of [^{3}H]ryanodine (6·25 nM). Incubation with 0·5 mg ml^{-1} muscle membrane protein at 27°C. $r^2 = 0{\cdot}999$. (b) Association of [^{3}H]isradipine (4·35 nM). Incubation with 0·5 mg ml^{-1} muscle membrane protein at 30°C. $r^2 = 0{\cdot}96$. (c) Association of [^{3}H]verapamil (50·49 nM). Incubation with 0·5 mg ml^{-1} muscle membrane protein at 30°C. $r^2 = 0{\cdot}97$. Inset: a linear transformation of the time course of specific [^{3}H]radioligand binding to *Periplaneta americana* muscle membranes (B_e = binding at equilibrium; B = specific binding at different times). r^2: coefficient of correlation of least squares fitted straight lines; standard deviation of the slope given in Table 2.

dose-dependent killing effect was observed for ryanodine with an LD_{50} (lethal dose for 50% killing) of 12·2 μg ml^{-1} applied to the meat. Verapamil, diltiazem and isradipine, in concentrations ranging from 0·1 to 300 μg ml^{-1}, showed no biological activity under the given conditions. No mortality of adult *L. cuprina* was observed on filter discs impregnated with verapamil, diltiazem or isradipine in concentrations ranging from 0·1 to 300 μg ml^{-1}. Adult flies treated with ryanodine on filter discs impregnated with the compound were killed with an LD_{50} value of 18·46 μg ml^{-1}. *P. americana* were also sensitive to ryanodine. With dipping assays, no specific differences in sensitivity to ryanodine were observed between larval stages, the LD_{50} for both the second- and third-instar larvae being 14 μg ml^{-1}, with an LD_{50} of 9 μg ml^{-1} for adult cockroaches. The LD_{50} values were derived from plots of logarithmic dose versus probit percentage mortality. A feeding experiment with fifth-instar *P. americana* indicated an LD_{50} of 0·1 μg ml^{-1} per cockroach.

Experiments on the effect of ryanodine on acari, which had not been tested previously, indicated an LD_{50} of 1030 μg ml^{-1} for adult *A. hebraeum* dipped in ryanodine solution and an ED_{50} (effective dose for 50% efficacy) of 0·49 μg per tick for inhibition of egg laying in fully engorged *B. microplus* injected with ryanodine. Calcium channel blockers added to cattle blood at 100 μg ml^{-1} had no lethal effect on fleas (*C. felis*) feeding from this blood through parafilm membranes, but verapamil and diltiazem caused 57 and 19% mortality, respectively, under these conditions.

4 DISCUSSION

Muscle contraction and relaxation is regulated by the intrafibre Ca^{2+} concentration, which, in turn, is regulated by membranes, whereby the sarcoplasmatic reti-

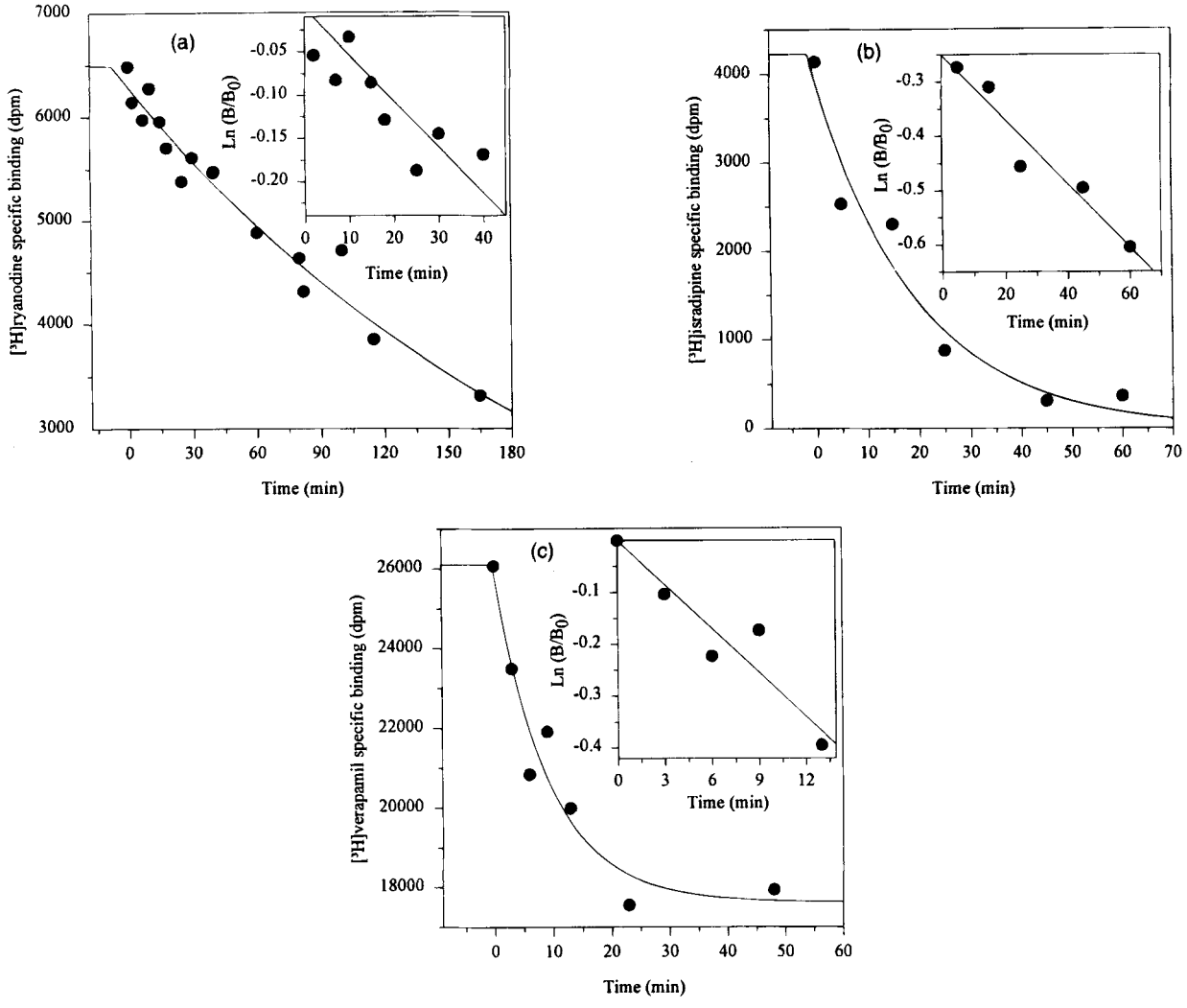

Fig. 4. Determination of dissociation rate constants. (a) Dissociation of [^{3}H]ryanodine (5 nM). Incubation with 0·5 mg ml^{-1} muscle membrane protein at 27°C. Subsequent dissociation brought about by addition of unlabelled ryanodine (5 μM) after 90 min. r^2 = 0·83 (b) Dissociation of [^{3}H]isradipine (4·7 nM). Incubation with 0·5 mg ml^{-1} muscle membrane protein at 30°C. Subsequent dissociation brought about by addition of unlabelled isradipine (5 μM) after 210 min. r^2 = 0·94. (c) Dissociation of [^{3}H]verapamil (29·2 nM). Incubation with 0·5 mg ml^{-1} muscle membrane protein at 30°C. Subsequent dissociation brought about by addition of unlabelled verapamil (50 μM) after 40 min. r^2 = 0·88. Inset: a linear transformation of the time course of specific [^{3}H]radioligand binding to *Periplaneta americana* muscle membranes (B = specific bound [^{3}H]radioligand retained at indicated times; B_0 = specific bound [^{3}H]radioligand at time 0). r^2: coefficient of correlation of least square fitted straight lines; standard deviation of the slope given in Table 2.

culum plays a key role in calcium uptake, storage and release.[16] Ryanodine, as a toxic alkaloid, binds specifically to the calcium-induced open state of the 'calcium release channel' present in the sarcoplasmatic reticulum of vertebrate and invertebrate muscle membranes, and which is involved in the release of calcium from sarcoplasmatic reticulum and leads to the contraction in this muscle.

The presence of a calcium channel with binding sites for 1,4-dihydropyridines and phenylalkylamines, similar to vertebrate L-type calcium channels, as well as the 'calcium release channel' (ryanodine receptor) was demonstrated in muscle membrane preparations of *P. americana*. Initial studies revealed evidence for a benzothiazepine receptor site in *P. americana* muscle membrane preparations as has previously been demonstrated with vertebrate L-type calcium channels.[17]

The presence of Ca^{2+} in incubation buffers plays an essential role in [^{3}H]ryanodine binding. Different studies have indicated that [^{3}H]ryanodine binds in a strictly Ca^{2+}-dependent manner.[18,19] Pessah *et al.*,[20] working with rabbit skeletal muscle membranes, observed an increase in ryanodine binding with increasing Ca^{2+} concentration up to 100 μM and decreased binding above this concentration. Our results with *P. americana* parallel these observations and confirm that the presence of Ca^{2+} at micromolar level can increase both affinity of the receptor for ryanodine and the apparent maximum binding capacity. Pre-incubation of muscle membrane protein with calcium and [^{3}H]ryanodine, followed by treatment with EGTA,

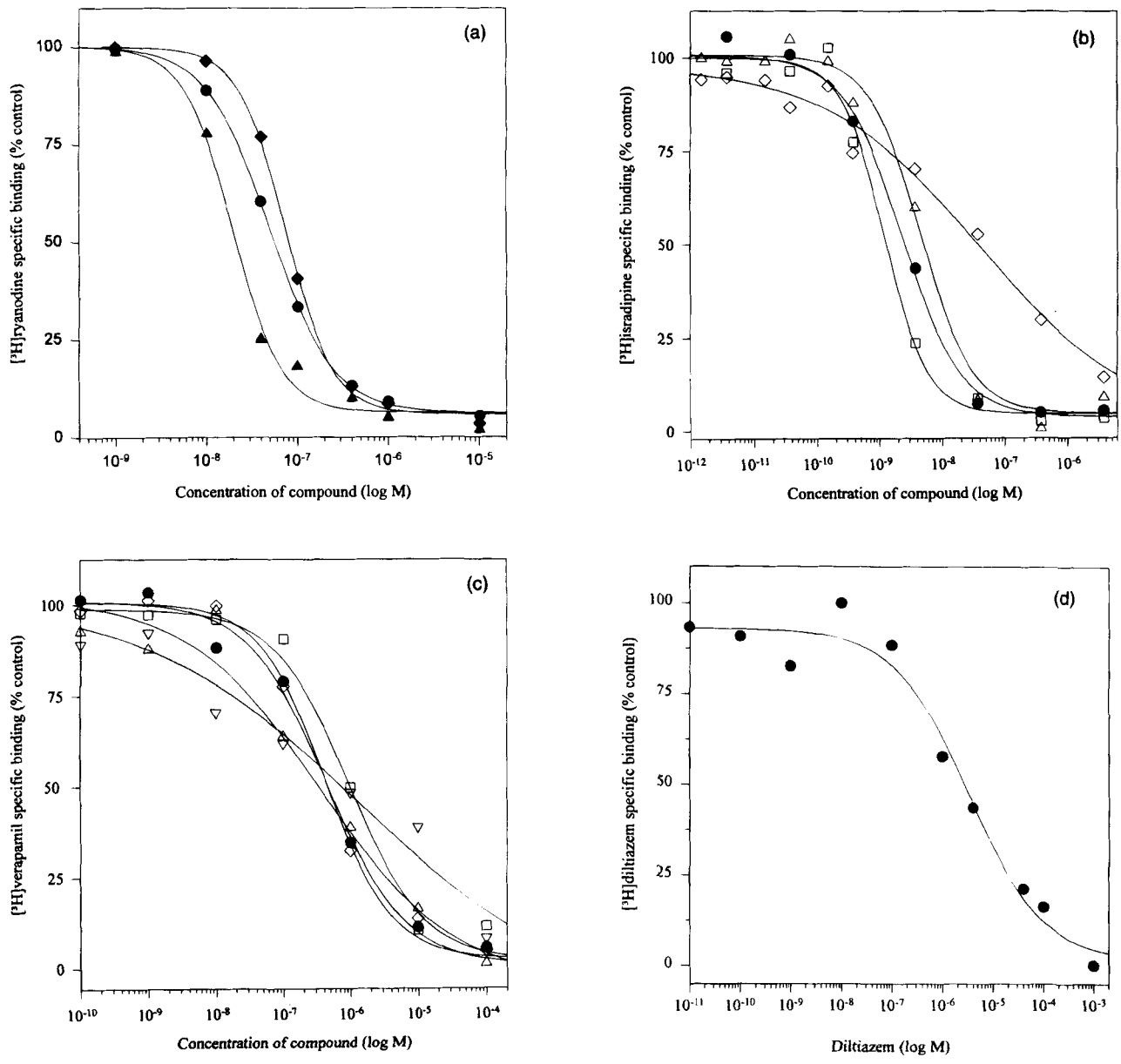

Fig. 5. Displacement curves with *Periplaneta americana* muscle membranes (0·5 mg ml^{-1}). Membrane protein was incubated with radioligand as described in Section 2.2.3 and competitors were added in concentrations given on the abscissa. (a) Displacement of specific [^{3}H]ryanodine binding (1 nM; 100% control binding corresponds to 3910 dpm), by (●) ryanodine mixture, (▲) pure ryanodine, (◆) 9·21 didehydroryanodine. (b) Displacement of specific [^{3}H]isradipine binding (37 nM; 100% control binding corresponds to 1040 dpm), by (●) isradipine, (□) nitrendipine, (△) Bay K8644, (◇) nicardipine. (c) Displacement of specific [^{3}H]verapamil binding (50 nM; 100% control binding corresponds to 1684 dpm) by (●) (±)verapamil, (▽) R(+)verapamil, (△) S(−)verapamil, (□) (±)nor-methylverapamil, (◇) bepridil. (d) Displacement of specific [^{3}H]diltiazem binding (70 nM; 100% control binding corresponds to 518 dpm) by (●) diltiazem. Standard deviation of the calculated four-parameter logistic curve fitting is given in Table 3. Standard deviation of the triplicate determination did not exceed 10% of the mean value.

revealed no pronounced effect on ryanodine binding even at concentrations of EGTA up to 300 μM. This result suggests that Ca^{2+} binds with a higher affinity to calcium-sensitive regions or Ca^{2+} binding sites at the ryanodine receptor than to EGTA. With EDTA, another chelator for divalent cations with lower affinity towards Ca^{2+}, ten-fold higher concentrations were required to yield the same effect on specific ryanodine binding as EGTA. An additional effect might be due to the chelation of divalent cations like Mg^{2+} by EDTA as was discussed by Meissner.[21] MgATP, rather than free ATP, should be a major physiological regulator of the ryanodine receptor in vertebrates, since most nucleotides in cells are complexed with Mg^{2+}. Therefore, a reduction of the Mg^{2+} concentration by trapping the cation in complexes with EDTA might lead to a decrease in the specific [^{3}H]ryanodine binding, because free ATP leaves the ryanodine receptor in a less active stage.[21] However, there is, as yet, no clear evidence for such observations in insects.

TABLE 3

K_i and IC_{50} Values Calculated from Competition Assays with 'Calcium Release Channel' and Calcium Channel in Muscle Membrane Preparations of the Cockroach *Periplaneta americana* (Figs 5(a)–(d))

Tritiated compound	*Competitor*	K_i (M)	(*S.D.*)
[^{3}H]verapamil	(±)Verapamil HCl	$4{\cdot}5 \times 10^{-7}$	(0·4)
	R(+)verapamil HCl	$1{\cdot}5 \times 10^{-6}$	(0·9)
	S(−)verapamil HCl	$3{\cdot}7 \times 10^{-7}$	(1·4)
	(±)*Nor*-methylverapamil	$9{\cdot}7 \times 10^{-7}$	(1·9)
	Bepridil HCl	$4{\cdot}3 \times 10^{-7}$	(1·6)
[^{3}H]isradipine	Isradipine	$1{\cdot}6 \times 10^{-9}$	(0·2)
	Nitrendipine	$1{\cdot}0 \times 10^{-9}$	(0·2)
	Nicardipine HCl	$2{\cdot}2 \times 10^{-8}$	(0·7)
	R(+)-Bay K8644	$4{\cdot}0 \times 10^{-9}$	(0·4)
[^{3}H]diltiazem	Diltiazem HCl	$3{\cdot}3 \times 10^{-6}$*	(1·9)
[^{3}H]ryanodine	Ryanodine (mixture)	$5{\cdot}0 \times 10^{-8}$	(0·2)
	Ryanodine (pure)	$2{\cdot}0 \times 10^{-8}$	(0·4)
	9,21-Didehydroryanodine	$7{\cdot}5 \times 10^{-8}$	(0·5)

Methods described in Section 2 and Fig. 5.
* Value for diltiazem is IC_{50}value.

Binding studies at different calcium concentrations with isradipine, verapamil and diltiazem revealed differences between these binding sites. With isradipine, specific binding at 500 μM Ca^{2+} with standard incubation methods had the highest value, whereas a ten-fold increase and a reduction by chelation with EGTA reduced the specific [^{3}H]isradipine binding, by 40 and 49% respectively. Poor inhibitory properties of calcium at 1,4-DHP binding sites have also been described for vertebrate skeletal muscle calcium channels (IC_{50} = 65 mM).[22] A high calcium concentration of 5 mM has an effect on specific [^{3}H]diltiazem binding in *P. americana* muscle membrane preparations with 20 and 66% reduction, respectively, in comparison to specific binding in buffer without exogenous calcium. A strong inhibition of diltiazem binding by divalent cations has been demonstrated previously for vertebrate skeletal muscle membrane preparations.[22,23]

Many [^{3}H]ryanodine binding experiments have been carried out in high-salt medium.[11,19] Michalak *et al.*[24] postulated that the stimulation of ryanodine binding by a high concentration of salt (sodium or potassium chloride) was due to ionic strength. In contrast Ogawa and Harafujii[25] found the stimulatory effect of high salt concentration was due to osmolarity rather than ionic strength, because they found no difference in ryanodine binding when equiosmotic concentrations of sucrose, instead of salt, were present in the incubation buffer. Inui *et al.*[19] observed maximal binding at sodium chloride concentrations above 0·8 M, a similar result being obtained with potassium chloride. We confirm an increase of ryanodine binding when sodium chloride or potassium chloride is present in the incubation buffer for insect muscle membrane. For *P. americana* we also showed a further increase in ryanodine binding with potassium chloride in the incubation buffer at concentrations between one and two molar, which was not observed with sodium chloride. The importance of high potassium chloride concentration was discussed previously by Lehmberg and Casida[11] as a possibility for membrane and receptor stabilization.

Modification of the membrane and membrane proteins by enzymes or chemicals can provide clues to the structure of a binding site, especially whether the binding site is a protein and thereby fulfils another receptor criterion. It was demonstrated that pre-treatment with proteinase K totally abolished the specific [^{3}H]ryanodine binding in our *P. americana* membrane preparations. Several studies have revealed that the calcium channel binding sites are heat-sensitive. Glossmann *et al.*[26] reported for the 1,4-DHP binding site of guinea-pig brain membranes a reduction of specific binding of more than 50% after exposure of membrane protein to a temperature of 50°C. Similar results were obtained with 1,4-DHP binding sites in rabbit muscle membrane preparations.[27] In our experiments, pre-treatment of *P. americana* membrane protein at 60°C abolished about 90% of the specific isradipine binding. In contrast to the observations of Glossmann *et al.*,[26] who showed that treatment of the 1,4-DHP binding site with DTT had no effect on the binding, our binding tests revealed 31% inhibition of the specific [^{3}H]isradipine binding after treatment of *P. americana* muscle membrane protein with dithiothreitol. The decrease in specific [^{3}H]isradipine binding suggests that -SH groups or disulfide bridges might be essential for the formation of the 1,4-DHP binding site. Interestingly, the *P. americana* isradipine binding site shows some

selectivity with respect to the protease treatment chosen. Whereas proteinase K treatment totally abolished specific isradipine binding, no effect on binding was observed with trypsin. It might be speculated that the aminoacids arginine and lysine, which are cleavage sites of trypsin, are not present or accessible in domains important for dihydropyridine binding. In our binding studies with the verapamil binding site it was shown that specific [^{3}H]verapamil binding was highly sensitive to treatment with DTT and to treatment with either proteinase K or trypsin. No effect on specific [^{3}H]verapamil binding was observed after pretreatment with heat. Reynolds *et al.*[23] showed that the [^{3}H]verapamil binding to rat muscle microsomal membranes was abolished upon treatment with trypsin and heat. The sensitivity of the benzothiazepine binding sites to heat, trypsin, DTT and proteinase K has not been shown yet for *P. americana* muscle membrane protein. Together, these observations suggest that the binding sites for [^{3}H]isradipine, [^{3}H]verapamil and [^{3}H]diltiazem as well as the [^{3}H]ryanodine binding site are proteins.

Displacement studies with ryanodine, 1,4-DHP, phenylalkylamine and benzothiazepine-receptor-selective compounds have been performed to prove specificity, which is another important pharmacological criterion to describe a receptor binding site.

Only a few compounds are known to be ryanodine receptor effectors. Together with the generally used mixture of 9,21-didehydroryanodine and ryanodine, the pure components of the mixture were tested for their ability to displace [^{3}H]ryanodine binding. The K_i value of pure ryanodine is about four-fold lower than the K_i value of 9,21-didehydroryanodine. Lehmberg and Casida[11] reported IC_{50} values of somewhat higher affinity but with the same rank order of specificity.

Displacement studies with [^{3}H]isradipine binding to skeletal muscle membranes from *P. americana* revealed high-affinity binding of nitrendipine, isradipine and Bay K8644 with K_i values in the nM range. Similar results have been reported with 1,4-DHP binding sites in vertebrates. At present isradipine seems to be the most useful radiolabelled ligand for binding studies because it has a high affinity for the skeletal muscle dihydropyridine receptor ($K_D = 0{\cdot}2$ nM).[22] Displacement of isradipine binding to rabbit skeletal muscle also revealed IC_{50} values of 2 nM for nitrendipine and 1 nM for isradipine.[28] Displacement profiles for [^{3}H]nitrendipine and non-labelled nitrendipine showed K_i values of 1·4 nM for rabbit T-tubule membranes.[29] These observations lead to the conclusion that the 1,4-DHP compounds tested so far do not show significant pharmacological differences between vertebrate and invertebrate calcium channel 1,4-DHP binding sites.

The phenylalkylamine receptor is characterized by a high stereoselectivity, (−)enantiomers being much more potent than (+)enantiomers.[4] Skeer and Sattelle[6] observed a 44-fold difference in activity between (−)- and (+)-verapamil in muscle membrane of *P. americana*; IC_{50} values of $6{\cdot}82 \times 10^{-7}$ M and 30×10^{-6} M, respectively, were found. Our data from binding studies with the same organism are in good accordance with the observation that verapamil inhibits stereospecifically, with a higher potency for (−)- than for (+)-verapamil but indicate only a four-fold difference in affinity. In a study with *D. melanogaster* head membranes, a similar ranking in affinity of the two verapamil enantiomers was observed in binding studies with the phenylalkylamine ligand [*N*-methyl ^{3}H]-Lu 49888.[30] The effects of calcium channel antagonists on phenylalkylamine binding to mammalian membrane preparations revealed different values. With guinea-pig skeletal muscle membrane, Glossmann and Ferry[31] showed an inverse ranking of affinity for the two enantiomers of verapamil. In that report, the (+)enantiomer displayed an IC_{50} of 30 nM whereas (−)verapamil had an IC_{50} of 77 nM in contrast to the data obtained in our binding assays. Galizzi *et al.*[32] compared different drugs with regard to their ability to displace [^{3}H]verapamil binding at rabbit skeletal muscle membranes. In their rank order of efficiency of the different molecules, bepridil was the most potent inhibitor with an IC_{50} of 7×10^{-7} M, which corresponds to the K_i of $4{\cdot}3 \times 10^{-7}$ M obtained for bepridil displacing [^{3}H]verapamil binding at cockroach skeletal muscle membranes in our study.

Scatchard analysis revealed that the ryanodine receptor for *P. americana* is a high-affinity binding site. Another study demonstrated ryanodine receptors in muscle membranes from *P. americana* having about four-fold lower affinity, with a K_D of 4·4 nM.[11] However, experimental conditions of that binding assay differed from the method presented here. As shown in this paper, the pH of the incubation buffer has a significant impact on the ryanodine binding, e.g. binding at pH 7·4 was only 26% of that at pH 8. For vertebrate membrane preparations Pessah *et al.*[33] found a K_D value of 2·2 nM for the ryanodine receptor of rabbit skeletal muscle. The association rate constant ($5{\cdot}86 \times 10^6$ M^{-1} min^{-1}) determined by the same authors for the vertebrate muscle was similar to that with *P. americana* in this work.

A comparison between 1,4-DHP, phenylalkylamine and benzothiazepine receptors of mammalian and insect skeletal muscle membranes indicates similarity in dissociation constants. Pauron *et al.*[4] identified a binding site for [^{3}H]verapamil in *D. melanogaster* head membranes and found a high-affinity class of binding sites with a dissociation constant of 0·36 nM and B_{max} of 1·6 pmol mg^{-1} protein. Skeer and Satelle[6] identified low-affinity binding sites for verapamil with *P. americana* with a K_D of 377 nM (muscle membrane) and 273 nM (nerve tissue). They used a centrifugation method but without filtration and washing with ice-cold

buffer, and high dissociation rate constants might lead to loss of receptor-bound radioligand during time-consuming separation steps. Additionally, the omission of an intensive washing with ice-cold buffer after centrifugation of the membrane could lead to high non-specific values.[34] According to Glossmann and Ferry,[31] membrane-bound calcium channels of guinea-pig skeletal muscle have a K_D of 1·4 nM for the DHP [^{3}H]isradipine. Pelzer *et al.*,[5] working on the receptors for Ca^{2+} channel blockers in *Drosophila* brain membranes, identified a 1,4-DHP binding site for [^{3}H]isradipine with a K_D of 1·1 nM (B_{max} = 36 fmol mg^{-1} protein) which corresponds to the K_D of 0·75 nM found in *P. americana* muscle membrane protein in our work.

The diltiazem binding site in the muscle membrane of *P. americana* measured by competition binding curve had a low-affinity binding site with an IC_{50} of $3{\cdot}3 \times 10^{-6}$ M. Glossmann *et al.*[17] showed that [^{3}H]diltiazem binds to guinea pig skeletal muscle membrane in a reversible manner and with an equilibrium dissociation constant of 39 nM and that [^{3}H]diltiazem label was found at more binding sites when experiments were done at 2°C than at 30°C. From this, they concluded that there is a temperature dependence between high-affinity and low-affinity states of the [^{3}H]diltiazem binding sites. Preliminary experiments indicated a similar effect in *P. americana* muscle membrane preparations, which needs to be further investigated.

The work reported here indicates that ryanodine is an effective insecticide but that it is not selectively active against invertebrates. A number of different application methods were used. High efficacy against ticks (*B. microplus*) was achieved as a result of injecting engorged female ticks, a method hitherto untested. Good activity was achieved against cockroaches as a result of ingestion but, by contrast, dipping *A. hebraeum* acari was effective only at higher concentrations, possibly due to the hydrophobic, waxy cuticle acting as barrier to uptake. However ryanodine showed good activity in dipping tests with *P. americana* at all developmental stages. The LD_{50} values in the range of 0·5 μg per animal (tick injection) and 19 μg ml^{-1} (adult flies) suggest that ryanodine is a very efficient calcium release blocker in arthropods.

In their report of the structure–activity relationships for natural ryanoids and ryanodine derivatives as inhibitors of the 'calcium release channel' receptor and toxicants for mice and houseflies, Waterhouse *et al.*[10] found no significant difference in toxicity between mammalian and insect systems. In contrast, ryanodol, a derivative of ryanodine had low toxicity to vertebrates, but was a potent knock-down agent for a variety of insects. Are insect calcium channels susceptible to calcium channel blockers and do insect calcium channels offer new potential targets for the development of new insecticides? The effects of calcium channel blockers other than verapamil on insects have not been reported as yet. With verapamil the blockage of calcium channels might lead to lethality since, in feeding experiments Hall *et al.*[35] found a dose-dependent killing effect with 100% mortality for wild-type (*Drosophila*) flies at the highest verapamil concentration (15 mM = 7·3 mg ml^{-1}) tested. To support their observations the authors demonstrated that suppression of the expression of the α_1 sub-unit of the calcium channel by a point mutation, producing a premature stop codon, leads to embryonic lethality.[35] Our tests revealed no significant effect on ticks, cockroaches or flies in concentrations between 0·1 and 300 μg ml^{-1}. For fleas an LD_{50} value of about 100 μg ml^{-1} was found in our blood-feeding assay with verapamil.

In general, it should be possible to exploit calcium channel and calcium release channel binding sites for the development of pesticides, probably with selectivity towards invertebrates, either by expression of cloned single sub-units or by preparation of complete channels as part of membrane fractions. Up to now the binding assays established in this paper deal with known commercially available compounds which are specific for potent drug binding sites in vertebrates. Since calcium channels and calcium release channels are complex regulated ion channels, specifically invertebrate calcium channel regulating compounds might be found in extensive screening efforts.

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